Biochimica et Biophysica Acta 1833 (2013) 3386–3395

Contents lists available at ScienceDirect

Biochimica et Biophysica Acta

journal homepage: www.elsevier.com/locate/bbamcr

Lipocalin-2 elicited by advanced glycation end-products promotes the migration of vascular smooth muscle cells

Tae-Wook Chung [a,b,1], Hee-Jung Choi [a,1], Cheorl-Ho Kim [b], Han-Sol Jeong [a], Ki-Tae Ha [a,*]

[a] Division of Applied Medicine, School of Korean Medicine, Pusan National University, Yangsan, Gyeongsangnam-do 626-870, Republic of Korea
[b] Department of Molecular and Cellular Glycobiology, College of Natural Science, Sungkyunkwan University, Suwon, Kyungki-do 440-746, Republic of Korea

ARTICLE INFO

Article history:
Received 28 May 2013
Received in revised form 11 October 2013
Accepted 14 October 2013
Available online 19 October 2013

Keywords:
Advanced glycation end-products
Lipocalin-2
Human aortic smooth muscle cells
Migration
Invasion

ABSTRACT

Advanced glycation end-products (AGEs) play key roles in the development of diabetic vascular complications by activating the proliferation and migration of vascular smooth muscle cells. Here, we identified an increase of the migratory properties of human aortic smooth muscle cells (HASMC) through AGE-induced expression of lipocalin-2 (LCN2). Because the AGE-elicited expression of LCN2 was diminished by an antibody against the AGE receptor (RAGE), diphenylene iodonium (DPI), N-acetyl cysteine, LY294002, and SP600125, we suggest that AGEs enhance the expression of LCN2 via a RAGE-NADPH oxidase-reactive oxygen species pathway, leading to the phosphorylation of PI3K-Akt and JNK in HASMCs. In addition, a chromatin immunoprecipitation assay and promoter assay revealed that CCAAT/enhancer binding protein β is crucial for AGE-induced expression of LCN2. However, any other AGE-related signaling pathway, including ERK1/2, p38, NF-κB, and AP-1, did not affect the AGE- induced expression of LCN2. Knockdown of LCN2 expression by shRNA showed that AGE-elicited LCN2 expression enhanced the invasive and migratory properties of HASMCs, but showed no effect on cell proliferation. Considering the importance of HASMC migration in the development of atherosclerosis, our study provides a novel insight into diabetic vascular complications.

1. Introduction

Atherosclerosis plays a pivotal role in cardiovascular morbidity and mortality in diabetic patients by causing coronary artery disease, stroke, and diabetic foot ulcers [1]. Although diverse pathologic factors have been implicated in the cardiovascular complications of diabetes, advanced glycation end-products (AGEs), which are the products of non-enzymatic glycation of proteins, are known as key players in the development of diabetic vascular complication [2]. AGEs bind to diverse receptors, including the receptor for AGEs (RAGE), class A, class B, type I and type II scavenger receptors, CD36, AGE receptor 1, AGE receptor 2, and galectin-3 [3–5]. RAGE is mainly associated with increases in oxidative stress, growth, and inflammatory effects, whereas the other receptors are involved in AGE detoxification, negative regulation of RAGE effects, and supporting inflammation [3,4,6]. AGEs accumulate in the plasma and increase vascular inflammation by interacting with RAGE on fibroblasts, hepatic stellate cells, monocytes, vascular endothelial cells, and vascular smooth muscle cells (VSMCs) [5,7–10]. Numerous studies have reported that AGEs induce the proliferation [7,11], calcification [12,13], adhesion [14], and migration [15] of VSMCs. Among the pathological processes related to atherosclerosis, VSMC migration into the intima plays an important role in the initial step of plaque formation [16]. Although the migratory activities of VSMCs are regulated by AGEs [17], the molecular mechanisms underlying this phenomenon are not fully understood.

Lipocalin-2 (LCN2) is also known as neutrophil gelatinase-associated lipocalin, and siderocalin is a 25-kDa glycoprotein belonging to the lipocalin family [18]. Because LCN2 binds to small lipophilic molecules such as bacteria-derived formylpeptides, leukotriene [19], and catechol-type siderophores [20], it was initially considered as a modulator of the innate immune system. In addition, LCN2 is involved in allosteric activation and degradation protection of matrix metalloproteinase (MMP)-9 [21,22]. Recent studies suggest that LCN2 also plays an important role in cell homeostasis, including morphogenesis [23], epithelial-to-mesenchymal transition [24], iron trafficking [25], and tumor growth [26]. Previous studies of LCN2 have been focused on its role as a biomarker in diverse diseases, including anemia [27], metabolic disease [28,29], inflammatory bowel disease [30], acute renal injury [31,32], and diabetic nephropathy [33–36]. In addition, elevated LCN2 expression is related to several vascular diseases, including atherosclerosis [37,38], myocardial infarction [38], and plaque instability [39]. However, the association of LCN2 with diabetic vascular complications is still unclear.

Here, we report for the first time that AGEs induce LCN2 expression in human aortic smooth muscle cells (HASMCs) through activation of CCAAT/enhancer binding protein β (C/EBPβ), but not NF-κB. In addition,

* Corresponding author at: Division of Applied Medicine, School of Korean Medicine, Pusan National University, Mulegum-eup, Yangsan, Gyeongsangnam-do 626-870, Republic of Korea. Tel.: +82 51 510 8464; fax: +82 51 510 8620.
E-mail address: hagis@pusan.ac.kr (K.-T. Ha).
[1] These authors equally contributed to this study.

0167-4889/$ – see front matter © 2013 Elsevier B.V. All rights reserved.
http://dx.doi.org/10.1016/j.bbamcr.2013.10.011

we suggest that AGE-elicited expression of LCN2 is crucial for enhancing the migration and invasion of HASMCs.

2. Materials and methods

2.1. Materials

Antibodies against the p65 subunit of NF-κB (F-6), c-fos (D-1), c-jun (H-79), C/EBP-β (C-19), lamin B (C-5), RAGE (A11), paxillin (H-114), and vinculin (H-300) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). An antibody against LCN2 (ab23477) was purchased from Abcam (Cambridge, MA). BAY 11-7082 (NF-κB inhibitor) and an antibody against β-actin was purchased from Sigma–Aldrich (St. Louis, MO). Inhibitors of specific signaling pathways, including LY294002 (PI3K inhibitor), U0126 (ERK1/2 inhibitor), SB203580 (p38 inhibitor), and SP600125 (JNK inhibitor), were purchased from Merck Millipore (Billerica, MA). All other chemicals and reagents, including human serum albumin (HSA) and glycated human albumin (AGEs), were obtained from Sigma–Aldrich unless otherwise indicated.

2.2. Cell culture

HASMCs were purchased from BioWhittacker (San Diego, CA) and cultured in smooth muscle cell growth medium-2 (SmGM-2) containing 10% fetal bovine serum (FBS; Sigma-Aldrich), 2 ng/mL human basic fibroblast growth factor, 0.5 ng/mL human epidermal growth factor, 50 mg/mL gentamicin, 50 mg/mL amphotericin-B, and 5 mg/mL bovine insulin. For all experiments, early passage HASMCs were grown to 80–90% confluence and then quiescence was induced by serum starvation (0.1% FBS) for at least 24 h.

2.3. Gelatin zymography

Cells were grown in serum-free medium with the indicated concentrations of AGEs for 24 h. Conditioned media were prepared in a sample buffer containing 62.5 mM Tris–HCl (pH 6.8), 10% glycerol, 2% SDS, and 0.00625% (w/v) bromophenol blue without boiling. The samples were loaded in an acrylamide/bisacrylamide (29.2:0.8) separating gel containing 0.1% (w/v) gelatin, and then electrophoresed at 4 °C. The gels were soaked twice in 0.25% Triton X-100 for 30 min at room temperature and then incubated at 37 °C for 20 h in incubation buffer containing 50 mM Tris–HCl (pH 7.6), 20 mM NaCl, 5 mM $CaCl_2$, and 0.02% Brij-58. The gels were then stained for 30 min in 0.1% (w/v) Coomassie blue R-250 solubilized in 30% methanol and 10% acetic acid.

2.4. Reverse transcription-polymerase chain reaction (RT-PCR) and quantitative real-time RT-PCR (qRT-PCR)

Total RNA was extracted from cells using TRIzol (Invitrogen, Carlsbad, CA). One microgram of total RNA from each sample was then subjected to reverse transcription with oligo-dT primers by using AccuPower RT-PreMix (Bioneer, Daejeon, Korea). The cDNA was amplified by PCR using AccuPower PCR-PreMix (Bioneer). The primers used to amplify *LCN2* and β-actin are shown in Supplementary Table 1. Amplified DNA was separated on 1.5% agarose gels and visualized under UV light. qRT-PCR was performed using iTaq™ Universal SYBR® Green Supermix (Bio-Rad) on a Bio-Rad iQ™ 5 Multicolor Real-Time PCR Detection System according to the manufacturer's instructions. Primer sequences for qRT-PCR were as follows: *LCN2*, forward 5′-TGATCCCAGCCCCACCT-3′ and reverse 5′-CCACTTCCCCTGGAATTGGT-3′; *β-actin*, forward 5′-CAAGAGATGGCCACGGCTGCT-3′ and reverse 5′-TCCTTCTGCATCCTGT CGGCA-3′.

2.5. Western blot analysis

Protein extracts from harvested cells were prepared using RIPA buffer (Cell Signaling, Danvers, MA) containing a protease inhibitor cocktail tablet (Roche, Mannheim, Germany). Nuclear proteins were extracted using an NE-PER Nuclear and Cytoplasmic Extraction Reagents Kit (Pierce, Rockford, IL). Equal quantities of proteins were size fractionated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and then electrotransferred to nitrocellulose membranes. The membranes were incubated with target protein-specific primary antibodies and horseradish peroxidase-conjugated secondary antibodies. After incubation, specific bands of interest were detected by ECL Plus (GE Healthcare, Uppsala, Sweden).

2.6. Cell growth determination

HASMCs were seeded in 6-well culture plates at a density of 1×10^6 cells per well in SmGM-2 supplemented with 10% FBS and then cultured for 24 h. The cells were treated with or without AGEs (200 μg/mL) for 72 h. Then, the cells were harvested, and the cell growth rates were determined using the Countess® Automated Cell Counter (Invitrogen).

2.7. Plasmid construction and luciferase assay

A 1-kb fragment of the *LCN2* promoter region was amplified from human genomic DNA by PCR and then cloned into a pGL4.17 vector (Promega, Fitchburg, WI). The sequence was verified by automated sequence analysis. Potential transcription factor binding sites were analyzed by Transcription Element Search System (TESS; http://www.cbil.upenn.edu/tess). Site-directed mutants of two different C/EBP sites were constructed using a QuikChange II XL site-directed mutagenesis kit (Stratagene, La Jolla, CA). The mutations were verified by sequence analysis. The primers for cloning and mutating the *LCN2* promoter are listed in Supplementary Table 1. The promoter plasmids were co-transfected with pCMV-β-galactosidase reporter plasmids (Clontech, Mountain View, CA) into HASMCs by using WelFect-EX™ PLUS reagent (WelGENE, Daegu, Korea). The cells were cultured for 24 h with medium containing 10% FBS, stimulated with AGEs for 24 h, and then collected and lysed by sonication in lysis buffer (25 mM Tris-phosphate, pH 7.8, 2 mM EDTA, 1% Triton X-100, and 10% glycerol). The cell lysates were assayed for luciferase activity using a luciferase assay kit (Promega) with normalization to β-galactosidase activity as the control.

2.8. Chromatin immunoprecipitation (ChIP) assay

ChIP was performed using a ChIP assay kit (Upstate, Utica, NY) according to the manufacturer's protocol. DNA-binding proteins were cross-linked with DNA and then incubated in SDS lysis buffer containing protease inhibitor cocktail tablet. DNA was sheared to 200–500 bp fragments by 30-s sonication pulses using a VC100 sonicator (Sonics & Materials, Newtown, CT). The chromatin solution was precleared with salmon DNA/protein Agarose 50% slurry (Upstate) for 30 min at 4 °C. The precleared supernatant was incubated overnight at 4 °C with anti-NF-κB, anti-C/EBP-β, anti-c-fos, and isotypic IgG antibodies. Precipitated DNA and input genomic DNA were amplified by PCR with primers shown in Supplementary Table 1. The PCR products were separated on a 2% agarose gel and visualized under UV light.

2.9. Knockdown of LCN2 by shRNA

Five human pLKO.1 lentiviral vectors encoding shRNAs targeting the *LCN2* gene were obtained from Open Biosystems (Thermo Scientific, Waltham, MA). Transfection was performed using WelFect-EX™ PLUS reagent (WelGENE) and selected with puromycin (2 μg/mL) to obtain 10 clones. The knockdown efficiency of the siRNAs against *LCN2*

expression was verified by gelatin zymography, RT-PCR, and western blot analysis.

2.10. Flow cytometric analysis of DNA content

Cells were harvested, centrifuged (2500 ×*g*, 4 °C, 10 min), and washed twice in PBS. The cell pellet was gently resuspended in 100 μL PBS, and 200 μL PBS containing 10% ethanol/5% glycerol was then added to the cells, followed by 200 μL PBS containing 50% ethanol/5% glycerol. The cell suspension was then incubated on ice for 5 min. One milliliter of PBS containing 70% ethanol/5% glycerol was added to the cell suspension, followed by incubation at 4 °C overnight. Then, the cell suspension was washed with PBS and resuspended with 250 μL of 1.12% sodium citrate buffer (pH 8.45) containing 12.5 μg RNase (Sigma–Aldrich). After further incubation at 37 °C for 30 min, cellular DNA was stained with 250 μL propidium iodide (50 μg/mL) for 30 min at room temperature. To assess the relative DNA content, the stained cells were analyzed by a FACSCanto II (BD Biosciences, San Jose, CA) with BD FACSDiva™ software.

2.11. Migration and invasion assays

The migration assay was performed using 24-well plates containing polycarbonate filter (8-μm pore size) inserts (Corning Inc., NY). For the invasion assay, the upper sides of the filters were coated with 100 μL of 0.2% gelatin. SmGM-2 containing 1% FBS with or without AGEs (200 μg/mL) was added to the lower chamber. HASMCs in SmGM-2 containing 1% FBS (5×10^4 cells/200 μL) were then added to the upper chambers in the presence or absence of AGEs (200 μg/mL). The cells were incubated at 37 °C with 5% CO_2 for 24 h. After incubation, the filter inserts were removed from the wells, and the cells on the upper side of the filter were removed using cotton swabs. The remaining cells on the filters were fixed, stained with hematoxylin and eosin, and mounted on microscope slides. The cells located on the underside of the filter were counted as migrating or invading cells.

2.12. Confocal microscopic observation

The cells maintained on glass coverslips were stimulated with AGEs (200 μg/mL). After 12 or 24 h, the cells were fixed with 4% paraformaldehyde in PBS and permeabilized with 0.2% Triton X-100 in PBS. After 1 h of incubation with blocking buffer (5% BSA in PBS), the cells were incubated with anti-C/EBPβ, -paxillin, or -vinculin antibodies (1:100) for 1 h, followed by 1 h of incubation with Texas Red-conjugated anti-rabbit IgG or fluorescein isothiocyanate (FITC)-conjugated anti-mouse IgG (1:1000; Invitrogen, Carlsbad, CA). After washing with cold PBS, actin was stained with Texas Red X phalloidin (Invitrogen), and the cells were mounted on glass slides using VECTASHIELD Mounting Medium (Vector Lab., Burlingame, CA). Fluorescent images were obtained using a ZEISS LSM700 laser scanning confocal microscope (Carl Zeiss, Oberkochen, Germany).

2.13. Statistical analysis

The data from proliferation, migration, invasion, and promoter assays are represented as the fold differences compared to the control and expressed as the mean ± SD. The differences between the mean values of 2 groups were determined by one-way analysis of variance with the post-hoc Dunnett's comparison. The minimum significance level was set at a *P* value of 0.05. All experiments were performed at least 3 times independently.

3. Results

3.1. AGEs enhance LCN2 expression, proliferation, and migration of HASMCs

AGEs have been reported to be an inducer of intimal hyperplasia [40] and VSMC migration [15], which is important in the pathogenesis of diabetes-related atherosclerosis. MMPs, especially MMP-2 and -9, have been reported to play key roles in the migration of VSMCs into the intima [41]. Thus, we considered that MMP-2 and -9 were, at least partially, responsible for the AGE-related migration of VSMCs. Surprisingly, the gelatin zymography data revealed that the expression of MMP-2 and -9 was not markedly changed by AGE treatment in HASMCs (Fig. 1A). The results from RT-PCR and western blot analyses of MMP-2 and -9 expression were the same as those obtained from gelatin zymography (data not shown). However, gelatin zymography showed that a protein band at about 130 kDa was increased by AGE treatment. In previous reports, this band was revealed to be a heterodimeric complex of MMP-9 and LCN2 [21,22]. Thus, we confirmed the expression of LCN2 mRNA and protein elicited by AGE treatment by using RT-PCR, western blot analysis, and qRT-PCR (Fig. 1A). Next, we examined the effect of AGEs on the proliferation and migration of HASMCs. The results revealed that AGEs significantly increased the proliferation and migration of HASMCs (Fig. 1B and C).

3.2. AGE-enhanced LCN2 expression is related to the RAGE-NADPH oxidase-reactive oxygen species (ROS) pathway, leading to activation of PI3K-Akt and JNK signal transduction in HASMCs

Circulating AGEs stimulate cells via binding to its receptor expressed on target cells, and subsequently activating NADPH oxidase activity and ROS production [9]. Thus, we examined whether this pathway is responsible for AGE-stimulated expression of *LCN2* by using specific antibodies or inhibitors. Our results showed that treatment with an anti-RAGE antibody, which was specific for the extracellular domain, diminished the expression of LCN2 mRNA and protein in AGE-treated HASMCs, but an isotypic anti-IgG antibody did not show such an effect (Fig. 2A). In addition, diphenylene iodonium (DPI), a known NADPH oxidase inhibitor, or *N*-acetyl cysteine, a ROS inhibitor, also decreased the expression of LCN2 mRNA and protein (Fig. 2B).

Mitogen-activated kinases (MAPKs) and PI3K-Akt are downstream signaling pathways of NADPH oxidase-ROS, and are involved in the proliferation and migration of VSMCs [7,42]. Therefore, we examined the relationship of these signaling molecules, including Akt, JNK, ERK, and p38, with AGE-induced expression of LCN2 mRNA and protein in HASMCs. Our data showed that AGEs increased the phosphorylation of these molecules (Fig. 2C and D). However, as shown in Fig. 2E, treatment with specific inhibitors showed that activation of Akt and JNK, but not ERK or p38, was required for AGE-induced expression of LCN2 mRNA and protein. In addition, treatment with BAY 11-7082, a specific inhibitor of NF-κB, also showed no effect on LCN2 expression.

3.3. Transactivation of C/EBPβ is responsible for AGE-induced LCN2 expression

Previous studies [18,43] and our analysis by TESS indicate NF-κB, AP-1, and C/EBPβ binding sites in the promoter region of the human *LCN2* gene (Fig. 3A). As shown in Fig. 2D, Akt and JNK were activated from 30 min to 3 h after AGE treatment. Thus, we examined nuclear translocation of the p65 subunit of NF-κB, c-fos, c-jun, and C/EBPβ. The data revealed that the nuclear translocation of C/EBPβ was remarkably increased from 3 to 6 h after AGE treatment. Translocation of p65 was slightly increased from 1 h, but localization of c-jun and c-fos was almost unchanged by AGE treatment (Fig. 3B). Furthermore, immunofluorescence staining for C/EBPβ showed that AGEs clearly induced translocation of C/EBPβ into the nucleus (Fig. 3C).

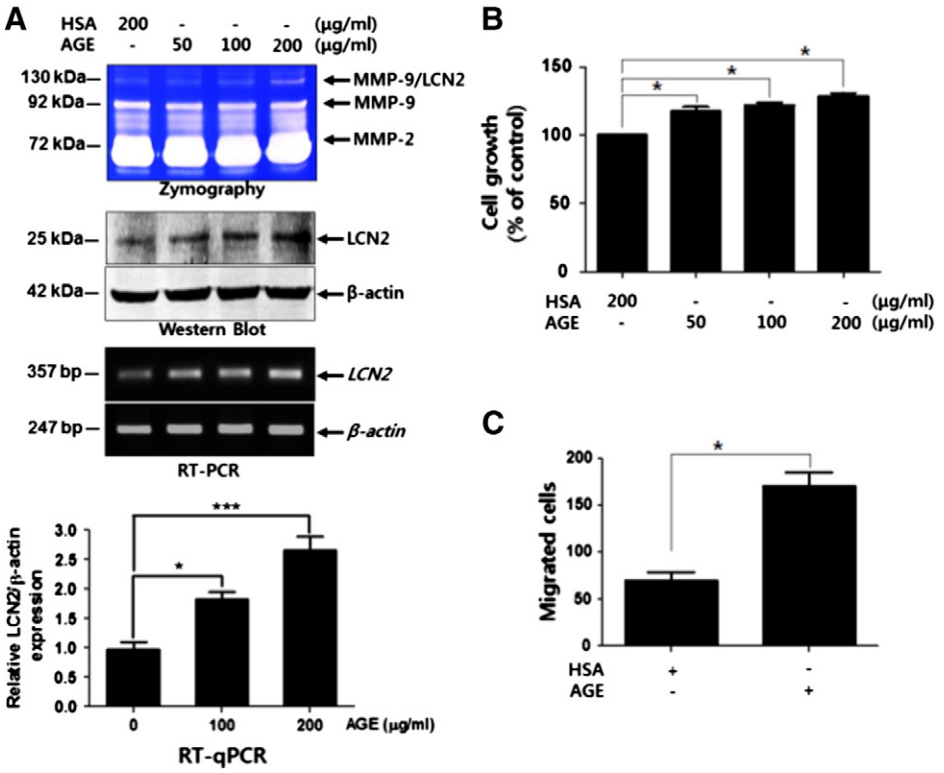

Fig. 1. ***AGEs enhance LCN2 expression, proliferation, and migration of HASMCs.*** The HASMCs were treated with HSA or the indicated concentrations of AGEs in serum-free medium for 24 h. (A) The supernatants were collected for gelatin zymography and total RNA and proteins were extracted from HSA- or AGE-treated HASMCs. Heterodimer formation of secreted LCN2 with MMP-9 was determined by gelatin zymography, and LCN2 expression was measured by western blot analysis, RT-PCR, and qRT-PCR. $^{*}P < 0.05$ and $^{***}P < 0.001$ vs. the HSA-treated control. (B) The growth rates of HSA- or AGE-treated HASMCs were estimated using a Countess Automated Cell Counter. $^{*}P < 0.05$ vs. the HSA-treated control. (C) HASMCs were cultured in the upper transwell chamber and treated with HSA (200 μg/mL) or AGEs (200 μg/mL) in serum-free medium for 24 h. The migrated cells on the underside of the transwell membrane were counted under a microscope. $^{*}P < 0.05$ vs. the HSA-treated control.

Next, we confirmed whether C/EBPβ is responsible for the transcriptional activation of *LCN2*. ChIP assay data revealed that the binding of C/EBPβ to the *LCN2* promoter was remarkably increased by AGE treatment. However, the binding affinity of p65 and c-fos was not increased by AGE treatment (Fig. 3D). The promoter assay also showed that the transcriptional activity of the *LCN2* promoter was significantly increased by AGE treatment (Fig. 3E). To investigate which of the 2 C/EBPβ binding sites were responsible for these observations, we performed the promoter assay using site-directed mutants of the C/EBPβ binding sites. The results showed that the C/EBP-2 site was responsible for transcriptional activation of AGE-stimulated *LCN2* expression in HASMCs (Fig. 3F). In addition, to verify whether C/EBPβ functions as a transcription factor for *LCN2* expression in AGE-treated HASMCs, we knocked down *C/EBPβ* gene expression using shC/EBPβ lentiviral vectors and then examined the *LCN2* promoter activity. As shown in Fig. 3G, AGEs affected transcriptional regulation of the *LCN2* gene via the C/EBPβ transcription factor.

3.4. Knockdown of LCN2 expression blocks AGE-elicited migration of HASMCs but does not affect proliferation

To investigate the biological function of *LCN2* elicited by AGE treatment, we knocked down *LCN2* gene expression using shLCN2 lentiviral vectors. Among 10 HASMC clones transfected with shLCN2, the knockdown efficiency of *LCN2* gene expression in 3 clones was confirmed by gelatin zymography, western blot analysis, RT-PCR, and qRT-PCR (Fig. 4A). We used a single clone, clone no. 6, for further experiments.

Because previous reports [7,11] and our data (Fig. 1B) showed that AGEs stimulate the proliferation and migration of HASMCs, we examined the possibility that the proliferation increase of AGE-treated HASMCs is related to *LCN2* expression. Our data showed that knockdown of *LCN2* expression did not affect AGE-stimulated proliferation of HASMCs (Fig. 4B). The results from the cell cycle analysis also did not show any significant change by *LCN2* gene knockdown (Fig. 4C). These results suggest that *LCN2* expression enhanced by AGE treatment is not responsible for the proliferation of HASMCs.

Previous studies have reported that *LCN2* expression is related to the migration and invasion of cancer cells by enhancing their motility and sustaining MMP-9 activity [44,45]. In addition, our results (Fig. 1C) and previous reports [42,46] showed that AGEs increase the migratory activity of HASMCs. Thus, we investigated the effect of AGE-induced LCN2 expression on the migration and invasion of HASMCs. Our results showed that knockdown of *LCN2* gene expression reduced the invasion and migration of AGE-stimulated HASMCs (Fig. 5A–C). Moreover, the results from confocal microscopic observation showed that shLCN2 suppressed AGE-induced recruitment of paxillin to focal adhesions and polymerization of F-actin (Fig. 5D). These data suggest that AGE-induced *LCN2* gene expression plays a crucial role in the invasion and migration of HASMCs (Fig. 6).

4. Discussion

AGEs resulting from the spontaneous non-enzymatic glycosylation of proteins are related to the removal of senescent proteins in normal aging processes [9,47]. However, excessive AGE formation is involved in the increase of atherosclerosis under a prolonged hyperglycemic condition [2,40]. Several reports have shown that AGEs increase the proliferation and migration of VSMCs through diverse signaling pathways [7,11,42,46]. The proteolytic activity of secreted MMPs regulates the

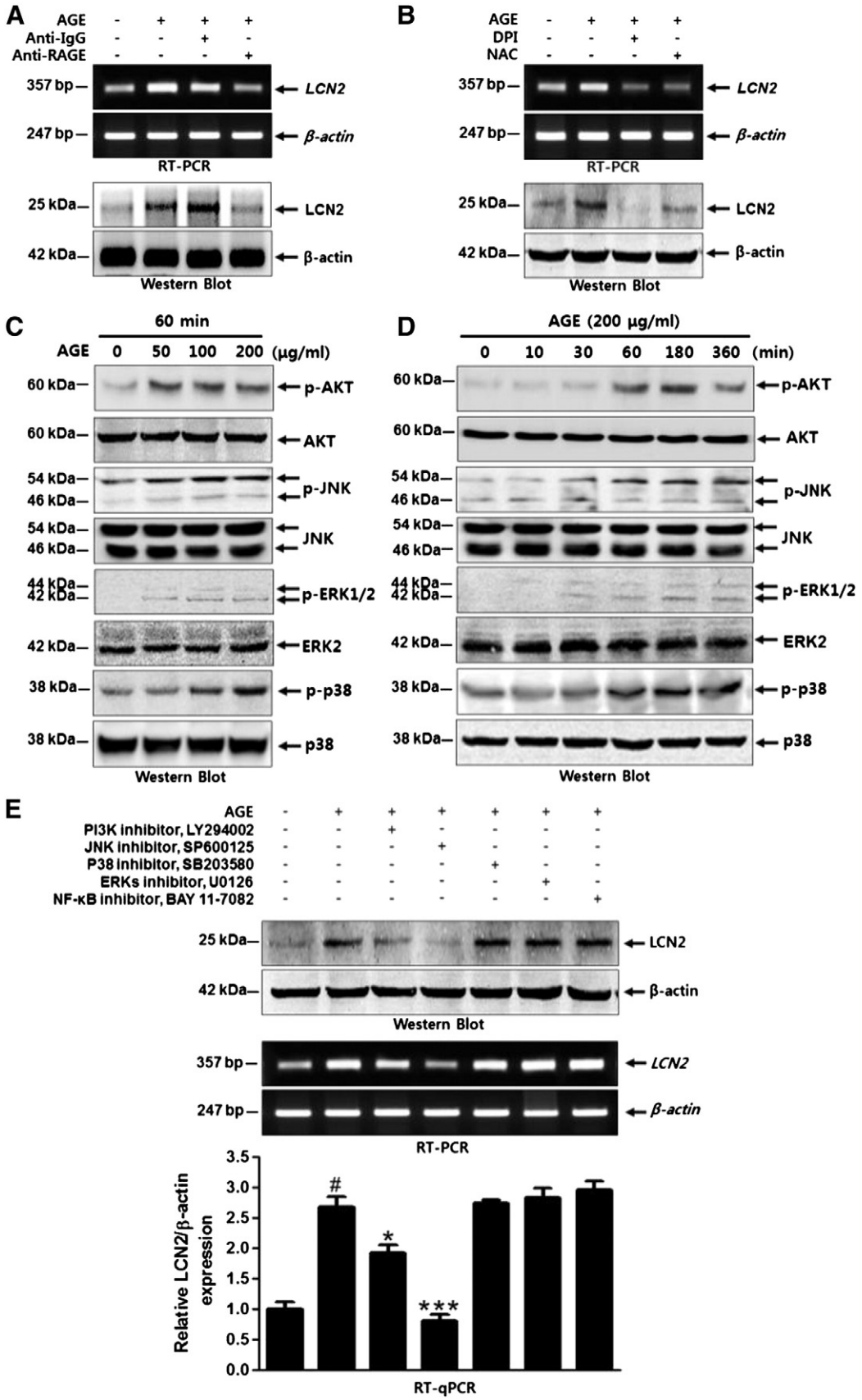

Fig. 2. ***Examination of the signaling pathways involved in AGE-induced LCN2 expression.*** (A, B) AGE-stimulated HASMCs were treated with an isotypic anti-IgG antibody, anti-RAGE antibody (A), DPI, or NAC (B) in serum-free medium for 24 h. HSA was used for the control. LCN2 expression was evaluated by RT-PCR and western blot analysis. (C, D) The phosphorylation of AGE-related signaling molecules, including Akt, JNK, ERK, and p38, was examined by western blot analysis. (E) HASMCs were treated with AGEs, LY294002, SP600125, SB203580, U0126, and/or BAY 11-7082. HSA was used as control. LCN2 expression was evaluated by western blot analysis, RT-PCR, and qRT-PCR. $^{\#}P < 0.05$ vs. the untreated control. $^{*}P < 0.05$ and $^{***}P < 0.001$ vs. the AGE-treated control.

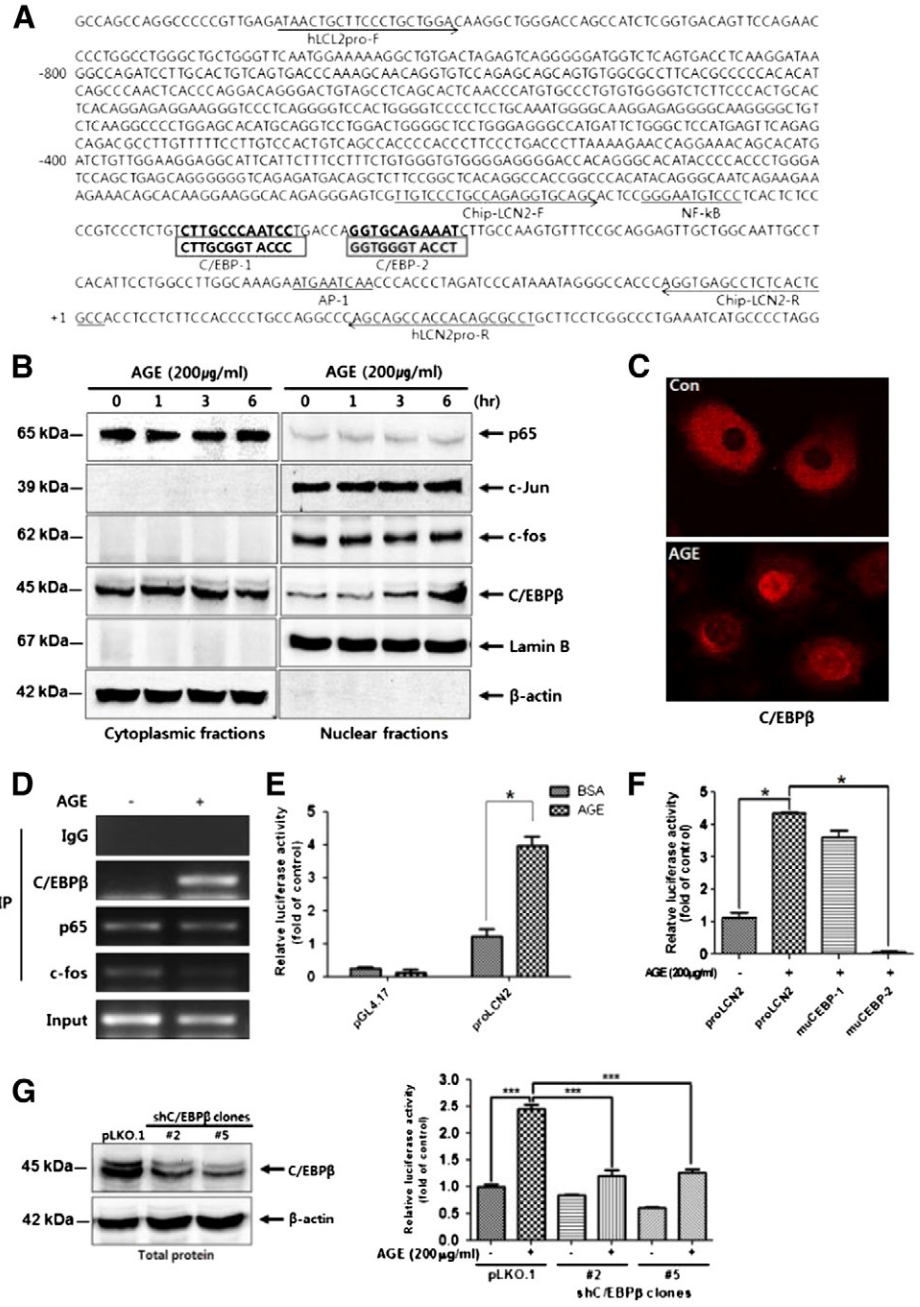

Fig. 3. ***Transcriptional activity of C/EBPβ on the LCN2 promoter as measured by ChIP and promoter assays.*** (A) The sequence from 1 kb upstream to 100 bp downstream of the human LCN2 transcription site (+1). Potential transcription factor binding sites are underlined and labeled, as identified by searching the TRANSFAC database. Boxed regions indicate two potential C/EBPβ binding sites. The sequences of the mutated C/EBPβ binding sites are shown under the box. The two primers pairs used for promoter cloning and ChIP assay are indicated by the arrowed underline. (B) After cytoplasmic and nuclear protein extraction, the nuclear translocation of p65, c-jun, c-fos, and C/EBPβ elicited by AGE stimulation was examined by western blot analysis. (C) HASMCs were treated with AGEs (200 μg/mL) for 12 h. The translocation of C/EBPβ into the nucleus was evaluated by immunofluorescence staining with an antibody against C/EBPβ. (D) HASMCs were treated with AGEs (200 μg/mL) for 24 h, and then cell lysates were subjected to a ChIP assay. DNA bound to C/EBPβ, the p65 subunit of NF-κB, and c-fos was precipitated with specific antibodies. An isotypic IgG was used for exclusion of nonspecific immunoprecipitation. Precipitated and input genomic DNAs were amplified by PCR. (E, F) HASMCs were cotransfected with a plasmid harboring different forms of the LCN2 promoter and pCMV-β-galactosidase reporter plasmids. The transfected cells were cultured for 24 h and then treated with AGEs. At 24 h after AGE stimulation, the cells were harvested for luciferase assays. $^{*}P < 0.05$ compared the indicated two groups. (G) The shC/EBPβ vector was introduced into HASMCs, followed by selection of clones. pLKO.1 vector-harboring HASMCs were used for the control. HASMCs harboring pLKO.1 or shC/EBPβ vector were cotransfected with a plasmid harboring different forms of the LCN2 promoter and pCMV-β-galactosidase reporter plasmids. The transfected cells were cultured for 24 h and then treated with AGEs. At 24 h after AGE stimulation, the cells were harvested for luciferase assays. $^{***}P < 0.001$ compared with the indicated two groups.

migration, proliferation, and survival of VSMCs [41]. In addition, AGEs enhance the expression of MMP-9 via activation of ERK, p38, and NF-κB in the murine macrophage cell line RAW264.7 and human keratinocyte HaCaT cells [48,49]. Thus, we presumed that AGEs might increase the expression and activity of MMPs, especially MMP-9, in HASMCs. However, gelatin zymography and RT-PCR assays showed that the expression and activity of MMP-2 and -9 were not influenced by AGE treatment in HASMCs. Interestingly, gelatin zymography results demonstrated that a protein band of about 130 kDa with gelatinolytic activity was increased by AGE treatment (Fig. 1A), indicating a heterodimer of LCN2 and MMP-9 corresponding to the band [21,22]. Furthermore, the results from RT-PCR and western blot analysis revealed

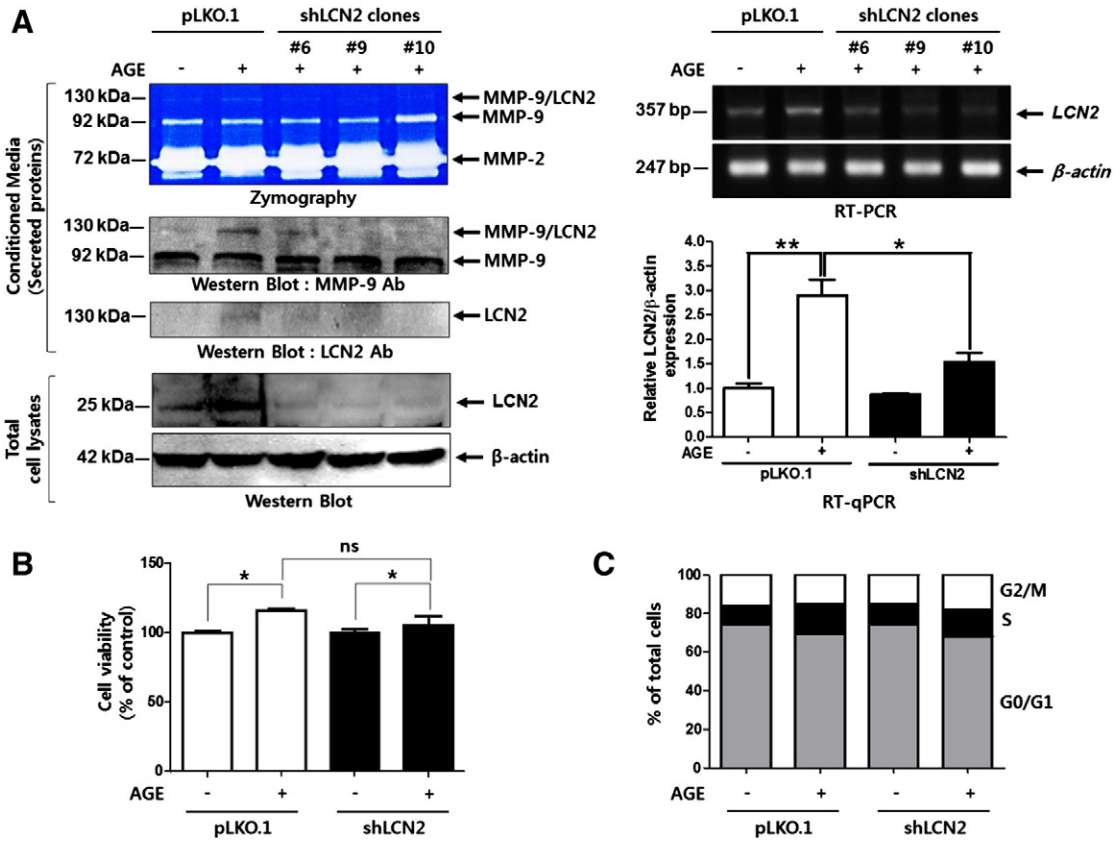

Fig. 4. ***shLCN2 inhibits LCN2 expression of AGE-stimulated HASMCs and does not influence cell proliferation.*** (A) The shLCN2 vector was introduced into HASMCs, followed by selection of clones. pLKO.1 vector-harboring HASMCs were used as control. LCN2 expression was evaluated by gelatin zymography, western blot analysis, RT-PCR, and qRT-PCR. $^{**}P<0.01$ vs. the HSA-treated control of the pLKO.1 group. $^{*}P<0.05$ vs. the AGE-treated control of the pLKO.1 group. (B) pLKO.1 and shLCN2 vector-harboring HASMCs were treated with HSA (200 μg/mL) or AGEs (200 μg/mL) for 24 h. The growth rates of each group were estimated using a Countess Automated Cell Counter. $^{*}P<0.05$ compared with the indicated two groups. ns, no significance. (C) The effect of shLCN2 on cell cycle regulation of AGE-stimulated HASMCs was estimated by DNA content analysis using flow cytometry.

that LCN2 expression was positively regulated (Fig. 1A), although the expression of MMP-9 was not changed by AGE treatment in HASMCs. On the other hand, methylglyoxal, the precursor of AGEs, had no effect on the expression of *LCN2* in HASMCs (Supplementary Fig. 1).

To elucidate the molecular mechanism underlying the AGE-elicited expression of *LCN2*, we examined AGE-related signaling pathways such as RAGE, NADPH oxidase-ROS, MAP kinases, PI3K-Akt, and NF-κB [7–9,13,42] using specific antibodies and signal inhibitors. The results showed that RAGE-NADPH oxidase-ROS was involved in AGE-induced *LCN2* expression (Fig. 2A and B). In addition, among the AGE-related downstream signaling molecules such as Akt, JNK, ERK, and p38, the phosphorylation of Akt and JNK was involved in *LCN2* expression. In particular, although NF-κB is the main transcription factor responsible for *LCN2* expression in the injured rat aorta [43], inhibition of NF-κB activity by a specific inhibitor, BAY 11-7082, had no effect on *LCN2* expression (Fig. 2C–E). Thus, we considered that there might be another transcription factor regulating LCN2 expression.

Previous studies have shown that about 1 kb upstream from the transcriptional start site is important for the regulation of *LCN2* expression, and the promoter region of *LCN2* has binding sites for diverse transcription factors such as NF-κB, AP-1, PU.1, and C/EBPβ [50–52]. In our analysis using the TRANSFAC database, NF-κB, AP-1, and two C/EBPβ binding sites were found in the core region of the *LCN2* promoter (Fig. 3A). Among the nuclear translocations of these transcription factors, only nuclear C/EBPβ was time-dependently increased by AGE treatment (Fig. 3B). In addition, the results from the ChIP assay and promoter assay showed that C/EBPβ is responsible for AGE-induced expression of LCN2 (Fig. 3C–E).

Although transcriptional activity of C/EBPβ is also regulated by other signaling molecules such as NF-κB, p38, and ERK [53,54], the expression of *LCN2* was regulated by transactivation of C/EBPβ through the phosphorylation of Akt and JNK in AGE-stimulated HASMCs. Therefore, the upregulation of C/EBPβ via activation of Akt and JNK might be involved in this phenomenon [55,56]. In addition, the signaling pathways involved in chronic inflammation elicited by AGEs may differ from those involved in acute inflammation, such as angioplastic vascular injury [43]. Further study is indeed to elucidate the precise mechanism underlying the differential activation of C/EBPβ.

AGEs enhance the progression of atherosclerosis by activating the migration, proliferation, and calcification of HASMCs [11,13,42,46]. The proliferation and migration of HASMCs were confirmed by the results shown in Fig. 1B and C. To elucidate the biological function of *LCN2* expression enhanced by AGE treatment, we knocked down *LCN2* gene expression by introducing an shRNA vector. The results shown in Figs. 4 and 5 indicated that shLCN2 decreased the migration, invasion, and motility of AGE-stimulated HASMCs, but did not influence their proliferation. Previously, *LCN2* was reported to be a positive regulator of proliferation in various malignant cells [18,57]. The reason for LCN2 showing no effect on the proliferation of AGE-treated HASMCs may be the tissue specificity or differences between benign and malignant cells. Alternatively, an elevation of ion channel activity [46,58] and expression of Pim1 [59] might be sufficient for AGE-induced proliferation of HASMCs. However, the results evidently showed that AGE-elicited expression of *LCN2* does not relate with the proliferation of HASMCs.

LCN2 forms a heterodimeric complex with MMP-9 [21] and sustains the gelatinolytic activity of MMP-9 by inhibiting autolytic degradation

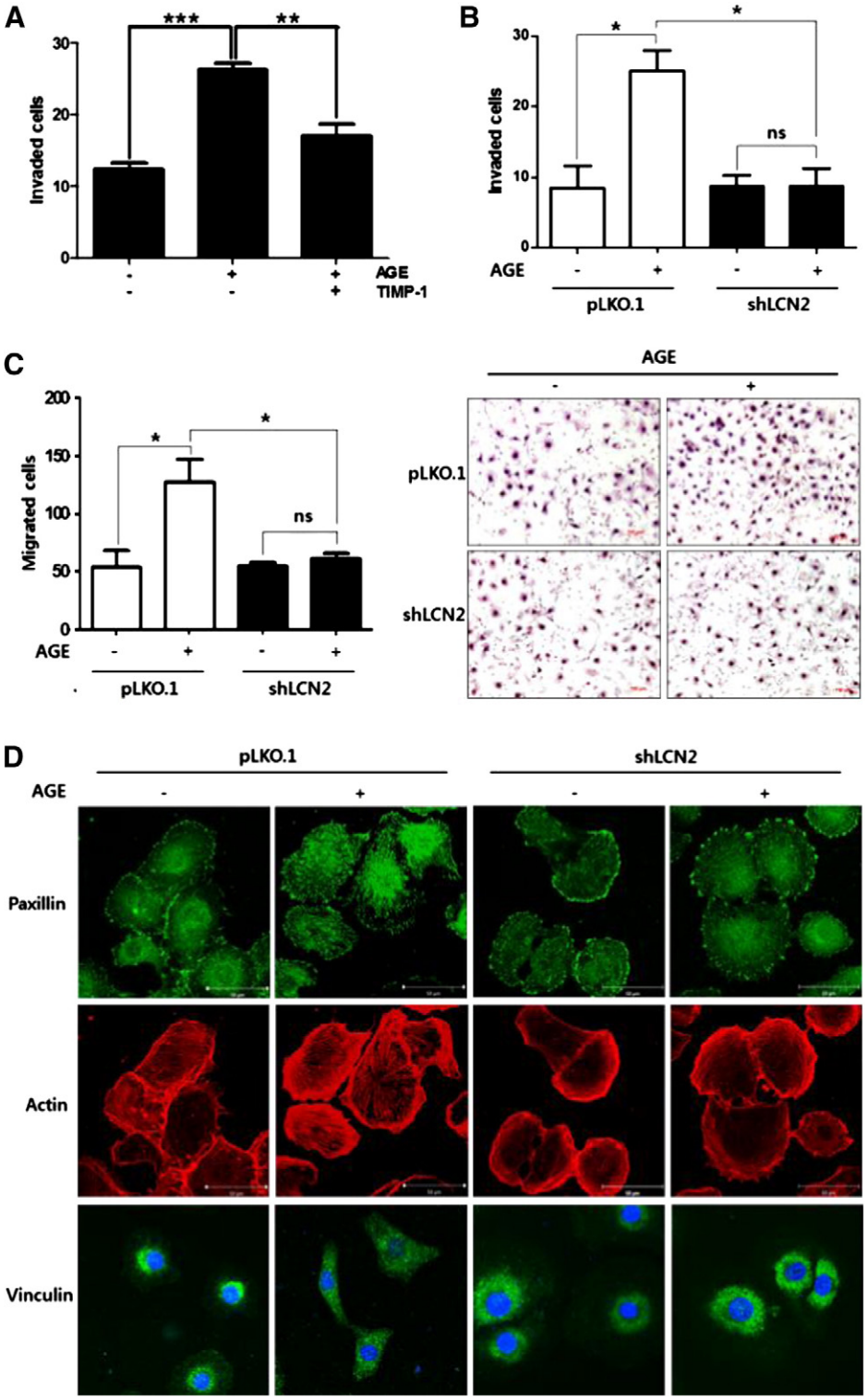

Fig. 5. ***Knockdown of LCN2 expression suppresses the migration and invasion of HASMCs.*** (A) HASMCs were seeded in the upper chamber of gelatin coated-transwells and treated with HSA (200 μg/mL), AGEs (200 μg/mL), or AGEs and an MMP-9 inhibitor, rhTIMP-1 (5 μg/mL), in serum-free medium for 24 h. The invaded cells on the underside of the transwell membrane were counted under a microscope. ***$P < 0.001$ vs. the HSA-treated control. **$P < 0.01$ vs. the AGE-treated control. (B) HASMCs harboring pLKO.1 or shLCN2 vectors were seeded in the upper chamber of gelatin coated-transwells and treated with HSA (200 μg/mL) or AGEs (200 μg/mL) in serum-free medium for 24 h. The invaded cells on the underside of the transwell membrane were counted under a microscope. *$P < 0.05$ compared with the indicated two groups. ns, no significance. (C) For migration assays, the transwell chamber was not coated with gelatin. The migrated cells on the underside of transwell membrane were counted under a microscope. * $P < 0.05$ compared with the indicated two groups. ns, no significance. Representative images of the migration assays are shown. (D) The effect of shLCN2 on the arrangement of paxillin, actin, and vinculin in AGE-stimulated HASMCs was observed by confocal microscopy. To detect paxillin and vinculin, the cells were permeabilized and incubated with anti-paxillin or anti-vinculin antibodies and then a FITC-conjugated secondary antibody (green). Actin was stained with phalloidin (red).

[22]. Thus, we considered that the increased invasiveness of LCN2-expressing HASMCs was related to the allosteric activation of MMP-9 activity. The enhanced invasiveness may be the result of other proteolytic enzymes, including cysteine proteases and serine protease [60,61]. However, the results from knockdown of *LCN2* expression by shRNA revealed that LCN2 plays an important role in the invasion of HASMCs

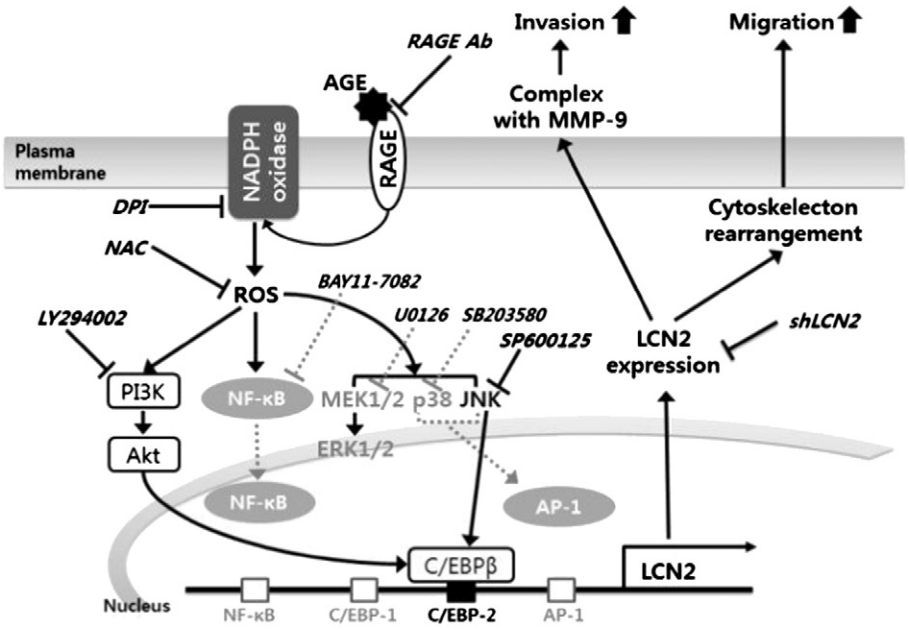

Fig. 6. ***Schematic representation of the effect of LCN2 on HASMC migration and the mechanism underlying AGE-induced LCN2 expression.*** The data presented in this study have shown that AGEs induce expression of LCN2 by activating a RAGE-NADPH oxidase-ROS pathway, leading to activation of the PI3K-Akt-C/EBPβ pathway and JNK-C/EBPβ pathway in HASMCs. We have also shown that inhibition of LCN2 expression by shLCN2 can block the AGE-induced invasive and migratory properties of HASMCs.

(Fig. 5A). In addition, elevated expression of *LCN2* activates the disassociation and motility of cancer cells as well as their invasiveness [44]. Our results and previous reports coordinately suggest that the increase of *LCN2* expression is responsible for the enhanced migration and motility of AGE-treated HASMCs (Fig. 5B–D). *LCN2* expression has been detected in atherosclerotic plaques, especially in macrophages [38,39]. However, *LCN2* expression has also been demonstrated in VSMCs and is especially high in intimal VSMCs [43]. Similar to the murine macrophage-like RAW264.7 cells, in the human monocytic cell line THP-1, MMP-9 expression is dose-dependently enhanced by AGE treatment [49]. However, AGEs did not enhance the secretion or expression of LCN2 in AGE-stimulated THP-1 cells (Supplementary Fig. 2). Thus, we suggest that LCN2 is mainly expressed and secreted from VSMCs in AGE-related vascular disease, thereby acting as an inducer of VSMC migration.

5. Conclusions

Our results indicate that AGEs induce the expression of *LCN2* by activating a RAGE-NADPH oxidase-ROS pathway, leading to activation of PI3K-Akt and JNK in HASMCs. We also found that transactivation of C/EBPβ is crucial for AGE-elicited *LCN2* expression. In addition, we have shown that inhibition of *LCN2* gene expression using shRNA can block the AGE-induced invasive and migratory properties of HASMCs. These results suggest that LCN2 is a novel and potent molecular target for diagnosis and treatment of diabetic vascular complications.

Conflict of interest

The authors have no conflicts of interest.

Acknowledgements

This study was supported by the Research Fund Program of the Research Institute for Basic Sciences, Pusan National University, Korea (Project No. RIBS-PNU-2012-112) and the Basic Science Research Program through the National Research Foundation of Korea (NRF), funded by the Ministry of Education, Science and Technology (2012R1A1A1005392).

Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.bbamcr.2013.10.011.

References

[1] A.K. Srivastava, High glucose-induced activation of protein kinase signaling pathways in vascular smooth muscle cells: a potential role in the pathogenesis of vascular dysfunction in diabetes (review), Int. J. Mol. Med. 9 (2002) 85–89.
[2] A. Goldin, J.A. Beckman, A.M. Schmidt, M.A. Creager, Advanced glycation end products: sparking the development of diabetic vascular injury, Circulation 114 (2006) 597–605.
[3] C. Lu, J.C. He, W. Cai, H. Liu, L. Zhu, H. Vlassara, Advanced glycation endproduct (AGE) receptor 1 is a negative regulator of the inflammatory response to AGE in mesangial cells, Proc. Natl. Acad. Sci. U. S. A. 101 (2004) 11767–11772.
[4] D. Neves, Advanced glycation end-products: a common pathway in diabetes and age-related erectile dysfunction, Free Radic. Res. 47 (Suppl. 1) (2013) 49–69.
[5] M. Alikhani, C.M. Maclellan, M. Raptis, S. Vora, P.C. Trackman, D.T. Graves, Advanced glycation end products induce apoptosis in fibroblasts through activation of ROS, MAP kinases, and the FOXO1 transcription factor, Am. J. Physiol. Cell Physiol. 292 (2007) C850–C856.
[6] J. Pang, D.H. Rhodes, M. Pini, R.T. Akasheh, K.J. Castellanos, R.J. Cabay, D. Cooper, M. Perretti, G. Fantuzzi, Increased adiposity, dysregulated glucose metabolism and systemic inflammation in Galectin-3 KO mice, PLoS One 8 (2013) e57915.
[7] H. Satoh, M. Togo, M. Hara, T. Miyata, K. Han, H. Maekawa, M. Ohno, Y. Hashimoto, K. Kurokawa, T. Watanabe, Advanced glycation endproducts stimulate mitogen-activated protein kinase and proliferation in rabbit vascular smooth muscle cells, Biochem. Biophys. Res. Commun. 239 (1997) 111–115.
[8] G. Basta, G. Lazzerini, S. Del Turco, G.M. Ratto, A.M. Schmidt, R. De Caterina, At least 2 distinct pathways generating reactive oxygen species mediate vascular cell adhesion molecule-1 induction by advanced glycation end products, Arterioscler. Thromb. Vasc. Biol. 25 (2005) 1401–1407.
[9] D.G. Farmer, S. Kennedy, RAGE, vascular tone and vascular disease, Pharmacol. Ther. 124 (2009) 185–194.
[10] E.L. Guimaraes, C. Empsen, A. Geerts, L.A. van Grunsven, Advanced glycation end products induce production of reactive oxygen species via the activation of NADPH oxidase in murine hepatic stellate cells, J. Hepatol. 52 (2010) 389–397.
[11] K.C. David, R.H. Scott, G.F. Nixon, Advanced glycation endproducts induce a proliferative response in vascular smooth muscle cells via altered calcium signaling, Biochem. Pharmacol. 76 (2008) 1110–1120.
[12] X. Ren, H. Shao, Q. Wei, Z. Sun, N. Liu, Advanced glycation end-products enhance calcification in vascular smooth muscle cells, J. Int. Med. Res. 37 (2009) 847–854.

[13] T. Tanikawa, Y. Okada, R. Tanikawa, Y. Tanaka, Advanced glycation end products induce calcification of vascular smooth muscle cells through RAGE/p38 MAPK, J. Vasc. Res. 46 (2009) 572–580.
[14] E. Hayakawa, T. Yoshimoto, N. Sekizawa, T. Sugiyama, Y. Hirata, Overexpression of receptor for advanced glycation end products induces monocyte chemoattractant protein-1 expression in rat vascular smooth muscle cell line, J. Atheroscler. Thromb. 19 (2012) 13–22.
[15] M.A. Reddy, S.L. Li, S. Sahar, Y.S. Kim, Z.G. Xu, L. Lanting, R. Natarajan, Key role of Src kinase in S100B-induced activation of the receptor for advanced glycation end products in vascular smooth muscle cells, J. Biol. Chem. 281 (2006) 13685–13693.
[16] S.M. Schwartz, Perspectives series: cell adhesion in vascular biology. Smooth muscle migration in atherosclerosis and restenosis, J. Clin. Invest. 99 (1997) 2814–2816.
[17] Y. Hattori, M. Suzuki, S. Hattori, K. Kasai, Vascular smooth muscle cell activation by glycated albumin (Amadori adducts), Hypertension 39 (2002) 22–28.
[18] S. Chakraborty, S. Kaur, S. Guha, S.K. Batra, The multifaceted roles of neutrophil gelatinase associated lipocalin (NGAL) in inflammation and cancer, Biochim. Biophys. Acta 1826 (2012) 129–169.
[19] T. Bratt, S. Ohlson, N. Borregaard, Interactions between neutrophil gelatinase-associated lipocalin and natural lipophilic ligands, Biochim. Biophys. Acta 1472 (1999) 262–269.
[20] D.H. Goetz, M.A. Holmes, N. Borregaard, M.E. Bluhm, K.N. Raymond, R.K. Strong, The neutrophil lipocalin NGAL is a bacteriostatic agent that interferes with siderophore-mediated iron acquisition, Mol. Cell 10 (2002) 1033–1043.
[21] H. Tschesche, V. Zolzer, S. Triebel, S. Bartsch, The human neutrophil lipocalin supports the allosteric activation of matrix metalloproteinases, Eur. J. Biochem. 268 (2001) 1918–1928.
[22] L. Yan, N. Borregaard, L. Kjeldsen, M.A. Moses, The high molecular weight urinary matrix metalloproteinase (MMP) activity is a complex of gelatinase B/MMP-9 and neutrophil gelatinase-associated lipocalin (NGAL). Modulation of MMP-9 activity by NGAL, J. Biol. Chem. 276 (2001) 37258–37265.
[23] J.A. Gwira, F. Wei, S. Ishibe, J.M. Ueland, J. Barasch, L.G. Cantley, Expression of neutrophil gelatinase-associated lipocalin regulates epithelial morphogenesis in vitro, J. Biol. Chem. 280 (2005) 7875–7882.
[24] R. Lim, N. Ahmed, N. Borregaard, C. Riley, R. Wafai, E.W. Thompson, M.A. Quinn, G.E. Rice, Neutrophil gelatinase-associated lipocalin (NGAL) an early-screening biomarker for ovarian cancer: NGAL is associated with epidermal growth factor-induced epithelio-mesenchymal transition, Int. J. Cancer 120 (2007) 2426–2434.
[25] G. Bao, M. Clifton, T.M. Hoette, K. Mori, S.X. Deng, A. Qiu, M. Viltard, D. Williams, N. Paragas, T. Leete, R. Kulkarni, X. Li, B. Lee, A. Kalandadze, A.J. Ratner, J.C. Pizarro, K.M. Schmidt-Ott, D.W. Landry, K.N. Raymond, R.K. Strong, J. Barasch, Iron traffics in circulation bound to a siderocalin (Ngal)-catechol complex, Nat. Chem. Biol. 6 (2010) 602–609.
[26] C.A. Fernandez, L. Yan, G. Louis, J. Yang, J.L. Kutok, M.A. Moses, The matrix metalloproteinase-9/neutrophil gelatinase-associated lipocalin complex plays a role in breast tumor growth and is present in the urine of breast cancer patients, Clin. Cancer Res. 11 (2005) 5390–5395.
[27] D. Bolignano, G. Coppolino, V. Donato, A. Lacquaniti, C. Bono, M. Buemi, Neutrophil gelatinase-associated lipocalin (NGAL): a new piece of the anemia puzzle? Med. Sci. Monit. 16 (2010) RA131–RA135.
[28] B.K. Tan, R. Adya, X. Shan, F. Syed, K.C. Lewandowski, J.P. O'Hare, H.S. Randeva, Ex vivo and in vivo regulation of lipocalin-2, a novel adipokine, by insulin, Diabetes Care 32 (2009) 129–131.
[29] Y. Wang, K.S. Lam, E.W. Kraegen, G. Sweeney, J. Zhang, A.W. Tso, W.S. Chow, N.M. Wat, J.Y. Xu, R.L. Hoo, A. Xu, Lipocalin-2 is an inflammatory marker closely associated with obesity, insulin resistance, and hyperglycemia in humans, Clin. Chem. 53 (2007) 34–41.
[30] D. Bolignano, A. Della Torre, A. Lacquaniti, G. Costantino, W. Fries, M. Buemi, Neutrophil gelatinase-associated lipocalin levels in patients with crohn disease undergoing treatment with infliximab, J. Investig. Med. 58 (2010) 569–571.
[31] R. Carrillo-Esper, F.M. Castillo-Albarran, J. Perez-Jauregui, Neutrophil gelatinase-associated lipocalin: a novel biomarker in acute kidney injury, Cir. Cir. 79 (2011) 577–581.
[32] A. Di Grande, C. Giuffrida, G. Carpinteri, G. Narbone, G. Pirrone, A. Di Mauro, S. Calandra, P. Noto, C. Le Moli, B. Alongi, F. Nigro, Neutrophil gelatinase-associated lipocalin: a novel biomarker for the early diagnosis of acute kidney injury in the emergency department, Eur. Rev. Med. Pharmacol. Sci. 13 (2009) 197–200.
[33] S.E. Nielsen, H.P. Hansen, B.R. Jensen, H.H. Parving, P. Rossing, Urinary neutrophil gelatinase-associated lipocalin and progression of diabetic nephropathy in type 1 diabetic patients in a four-year follow-up study, Nephron Clin. Pract. 118 (2011) c130–c135.
[34] D. Bolignano, A. Lacquaniti, G. Coppolino, V. Donato, M.R. Fazio, G. Nicocia, M. Buemi, Neutrophil gelatinase-associated lipocalin as an early biomarker of nephropathy in diabetic patients, Kidney Blood Press. Res. 32 (2009) 91–98.
[35] J. Malyszko, H. Bachorzewska-Gajewska, E. Sitniewska, J.S. Malyszko, B. Poniatowski, S. Dobrzycki, Serum neutrophil gelatinase-associated lipocalin as a marker of renal function in non-diabetic patients with stage 2-4 chronic kidney disease, Ren. Fail. 30 (2008) 625–628.
[36] Y.H. Yang, X.J. He, S.R. Chen, L. Wang, E.M. Li, L.Y. Xu, Changes of serum and urine neutrophil gelatinase-associated lipocalin in type-2 diabetic patients with nephropathy: one year observational follow-up study, Endocrine 36 (2009) 45–51.
[37] A.M. Elneihoum, P. Falke, B. Hedblad, F. Lindgarde, K. Ohlsson, Leukocyte activation in atherosclerosis: correlation with risk factors, Atherosclerosis 131 (1997) 79–84.
[38] A.L. Hemdahl, A. Gabrielsen, C. Zhu, P. Eriksson, U. Hedin, J. Kastrup, P. Thoren, G.K. Hansson, Expression of neutrophil gelatinase-associated lipocalin in atherosclerosis and myocardial infarction, Arterioscler. Thromb. Vasc. Biol. 26 (2006) 136–142.
[39] B.C. te Boekhorst, S.M. Bovens, W.E. Hellings, P.H. van der Kraak, K.W. van de Kolk, A. Vink, F.L. Moll, M.F. van Oosterhout, J.P. de Vries, P.A. Doevendans, M.J. Goumans, D.P. de Kleijn, C.J. van Echteld, G. Pasterkamp, J.P. Sluijter, Molecular MRI of murine atherosclerotic plaque targeting NGAL: a protein associated with unstable human plaque characteristics, Cardiovasc. Res. 89 (2011) 680–688.
[40] S. Yamagishi, T. Matsui, Smooth muscle cell pathophysiology and advanced glycation end products (AGEs), Curr. Drug Targets 11 (2010) 875–881.
[41] A.C. Newby, Matrix metalloproteinases regulate migration, proliferation, and death of vascular smooth muscle cells by degrading matrix and non-matrix substrates, Cardiovasc. Res. 69 (2006) 614–624.
[42] F. Toure, G. Fritz, Q. Li, V. Rai, G. Daffu, Y.S. Zou, R. Rosario, R. Ramasamy, A.S. Alberts, S.F. Yan, A.M. Schmidt, Formin mDia1 mediates vascular remodeling via integration of oxidative and signal transduction pathways, Circ. Res. 110 (2012) 1279–1293.
[43] D.X. Bu, A.L. Hemdahl, A. Gabrielsen, J. Fuxe, C. Zhu, P. Eriksson, Z.Q. Yan, Induction of neutrophil gelatinase-associated lipocalin in vascular injury via activation of nuclear factor-kappaB, Am. J. Pathol. 169 (2006) 2245–2253.
[44] L. Hu, W. Hittelman, T. Lu, P. Ji, R. Arlinghaus, I. Shmulevich, S.R. Hamilton, W. Zhang, NGAL decreases E-cadherin-mediated cell-cell adhesion and increases cell motility and invasion through Rac1 in colon carcinoma cells, Lab. Invest. 89 (2009) 531–548.
[45] C. Nuntagowat, K. Leelawat, R. Tohtong, NGAL knockdown by siRNA in human cholangiocarcinoma cells suppressed invasion by reducing NGAL/MMP-9 complex formation, Clin. Exp. Metastasis 27 (2010) 295–305.
[46] L.M. Zhao, X.L. Su, Y. Wang, G.R. Li, X.L. Deng, KCa3.1 channels mediate the increase of cell migration and proliferation by advanced glycation endproducts in cultured rat vascular smooth muscle cells, Lab. Invest. 93 (2013) 159–167.
[47] A.M. Schmidt, O. Hori, J. Brett, S.D. Yan, J.L. Wautier, D. Stern, Cellular receptors for advanced glycation end products. Implications for induction of oxidant stress and cellular dysfunction in the pathogenesis of vascular lesions, Arterioscler. Thromb. 14 (1994) 1521–1528.
[48] P. Zhu, M. Ren, C. Yang, Y.X. Hu, J.M. Ran, L. Yan, Involvement of RAGE, MAPK and NF-kappaB pathways in AGEs-induced MMP-9 activation in HaCaT keratinocytes, Exp. Dermatol. 21 (2012) 123–129.
[49] F. Zhang, G. Banker, X. Liu, P.A. Suwanabol, J. Lengfeld, D. Yamanouchi, K.C. Kent, B. Liu, The novel function of advanced glycation end products in regulation of MMP-9 production, J. Surg. Res. 171 (2011) 871–876.
[50] J.R. Karlsen, N. Borregaard, J.B. Cowland, Induction of neutrophil gelatinase-associated lipocalin expression by co-stimulation with interleukin-17 and tumor necrosis factor-alpha is controlled by IkappaB-zeta but neither by C/EBP-beta nor C/EBP-delta, J. Biol. Chem. 285 (2010) 14088–14100.
[51] J.B. Cowland, O.E. Sorensen, M. Sehested, N. Borregaard, Neutrophil gelatinase-associated lipocalin is up-regulated in human epithelial cells by IL-1 beta, but not by TNF-alpha, J. Immunol. 171 (2003) 6630–6639.
[52] J.B. Cowland, N. Borregaard, Molecular characterization and pattern of tissue expression of the gene for neutrophil gelatinase-associated lipocalin from humans, Genomics 45 (1997) 17–23.
[53] Y. Wang, R. Singh, Y. Xiang, L.E. Greenbaum, M.J. Czaja, Nuclear factor kappaB up-regulation of CCAAT/enhancer-binding protein beta mediates hepatocyte resistance to tumor necrosis factor alpha toxicity, Hepatology 52 (2010) 2118–2126.
[54] D.M. Cortez, M.D. Feldman, S. Mummidi, A.J. Valente, B. Steffensen, M. Vincenti, J.L. Barnes, B. Chandrasekar, IL-17 stimulates MMP-1 expression in primary human cardiac fibroblasts via p38 MAPK- and ERK1/2-dependent C/EBP-beta, NF-kappaB, and AP-1 activation, Am. J. Physiol. Heart Circ. Physiol. 293 (2007) H3356–H3365.
[55] M. Sachdeva, Q. Liu, J. Cao, Z. Lu, Y.Y. Mo, Negative regulation of miR-145 by C/EBP-beta through the Akt pathway in cancer cells, Nucleic Acids Res. 40 (2012) 6683–6692.
[56] Y.H. Cho, C.H. Lee, S.G. Kim, Potentiation of lipopolysaccharide-inducible cyclooxygenase 2 expression by C2-ceramide via c-Jun N-terminal kinase-mediated activation of CCAAT/enhancer binding protein beta in macrophages, Mol. Pharmacol. 63 (2003) 512–523.
[57] D. Bolignano, V. Donato, A. Lacquaniti, M.R. Fazio, C. Bono, G. Coppolino, M. Buemi, Neutrophil gelatinase-associated lipocalin (NGAL) in human neoplasias: a new protein enters the scene, Cancer Lett. 288 (2010) 10–16.
[58] S. Wu, T. Song, S. Zhou, Y. Liu, G. Chen, N. Huang, L. Liu, Involvement of Na+/H + exchanger 1 in advanced glycation end products-induced proliferation of vascular smooth muscle cell, Biochem. Biophys. Res. Commun. 375 (2008) 384–389.
[59] J. Meloche, R. Paulin, A. Courboulin, C. Lambert, M. Barrier, P. Bonnet, M. Bisserier, M. Roy, M.A. Sussman, M. Agharazii, S. Bonnet, RAGE-dependent activation of the oncoprotein Pim1 plays a critical role in systemic vascular remodeling processes, Arterioscler. Thromb. Vasc. Biol. 31 (2011) 2114–2124.
[60] B. Fuhrman, The urokinase system in the pathogenesis of atherosclerosis, Atherosclerosis 222 (2012) 8–14.
[61] X.W. Cheng, Z. Huang, M. Kuzuya, K. Okumura, T. Murohara, Cysteine protease cathepsins in atherosclerosis-based vascular disease and its complications, Hypertension 58 (2011) 978–986.